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Quantification of Mechanical Forces and Physiological Processes Involved in Pollen Tube Growth Using Microfluidics and Microrobotics

Jan Burri[1], Gautam Munglani[2], Bradley J. Nelson[1], Ueli Grossniklaus[2], Hannes Vogler[2*]

[1]Multi-Scale Robotics Lab, Institute of Robotics and Intelligent Systems, ETH Zurich, Zurich, Switzerland

[2]Department of Plant and Microbial Biology & Zurich-Basel Plant Science Center, University of Zurich, Zurich, Switzerland

*Corresponding author; email: hannes.vogler@botinst.uzh.ch

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Abstract

Pollen tubes face many obstacles on their way to the ovule. They have to decide whether to navigate around cells or penetrate the cell wall and grow through it or even within it. Besides chemical sensing, which directs the pollen tubes on their path to the ovule, this involves mechanosensing to determine the optimal strategy in specific situations. Mechanical cues then need to be translated into physiological signals, which eventually lead to changes in the growth behavior of the pollen tube. To study these events, we have developed a system to directly quantify the forces involved in pollen tube navigation. We combined a lab-on-a-chip device with a microelectromechanical systems-based force sensor to mimic the pollen tube's journey from stigma to ovary *in vitro*. A force-sensing plate creates a mechanical obstacle for the pollen tube to either circumvent or attempt to penetrate while measuring the involved forces in real-time. The change of growth behavior and intracellular signaling activities can be observed with a fluorescence microscope.

Key words: Pollen tube (PT), Growth, Force sensor, Perceptive force, Penetrative force, Lab-On-a-Chip (LOC), MicroElectroMechanical System (MEMS), Calcium (Ca^{2+}), Imaging, Fluorescence

1 Introduction

Pollen tubes (PTs) have to navigate a labyrinth of obstacles on their way to the ovule where they burst and release the sperm cells they carry to effect double fertilization. This requires a continuous adaptation of growth parameters, such as growth rate and orientation, which is accompanied by changes in physiological parameters, cytoskeletal organization, and cell wall properties (reviewed in: ***(1)***). For example, a tip-focused calcium (Ca^{2+}) gradient is necessary for PT growth ***(2-4)*** and could be involved in growth reorientation ***(5, 6)***. A growing number of molecular sensors, mostly genetically encoded fluorescent proteins, have been developed to visualize and quantify many of the intracellular factors involved ***(7)***.

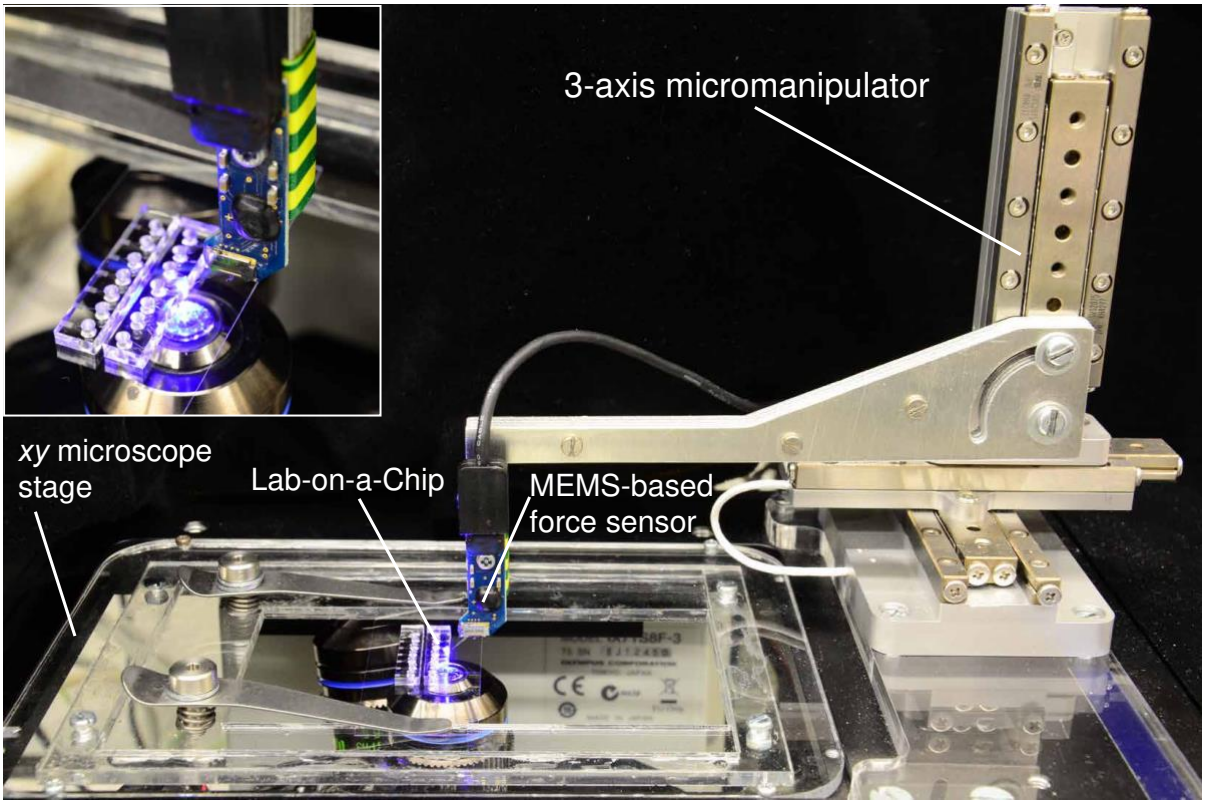

Fig. 1 System configuration to simulate the natural environment of the pollen tube in vitro with integrated force readout. It consists of a LOC device, a MEMS-based capacitive force sensor, and a 3-axis micropositioner mounted on a xy microscope stage of a fluorescence microscope. Close up view on the top left.

To simultaneously study the forces that are involved in PT growth and their effect on cellular parameters, we developed a system that combines a microfluidic Lab-On-a-Chip (LOC) device with a microrobotics system, i.e. a MicroElectroMechanical System (MEMS)-based force sensor (Fig. 1). The LOC consists of multiple microchannel systems and serves to parallelize the growth of multiple PTs confined to the same focal plane, which greatly facilitates force measurements and microscopic observation ***(8)***. Polydimethylsiloxane (PDMS) microchannels can be produced for a wide range of PT diameters and, if required, in many shapes (i.e. zigzag). Based on components of the Cellular Force Microscope (CFM), ***(9-17)*** the microrobotics system precisely positions the force-sensing microplate in front of the microchannels to wait for the PTs to emerge and to interact with it. With this setup, we found that PTs press against an obstacle until they reach a perceptive threshold force. Then, after a short lag phase, they reorient their growth direction ***(18)***.

To analyze the corresponding changes in the levels of physiological parameters or cytoskeletal organization in real time, fluorescent imaging at high spatial and temporal resolution is necessary. Especially, if quantification is based on ratiometric imaging, the use of a dual view camera system, which acquires both the donor and acceptor channels simultaneously, is advisable. For ratiometric systems with two different excitation wavelengths, a fast-switchable LED illumination system works well. Alternatively, fast filterwheels can be used in the excitation and/or emission light path.

Since individual measurements often take minutes or even hours, the resulting image stacks can contain hundreds or even thousands of frames. Manual handling of such large datasets is tedious if not impossible and, therefore, requires automatic image processing, including background subtraction and bleaching correction. If growth-related changes in tip-growing cells are to be analyzed, permanent tip tracking is of the essence.

Here, we present a protocol for quantifying the changes in the tip-based Ca^{2+} gradient when a PT grows into an obstacle, while simultaneously measuring the involved forces and changes in growth direction. For Ca^{2+} quantification we used the Förster Resonance Energy Transfer (FRET)-based ratiometric sensor Yellow Cameleon 3.60 (YC3.60) ***(19)***. We describe custom-made image processing and analysis toolkits that we developed for the efficient analysis of large datasets. As the LOCs used for this method are not commercially available, we also provide a protocol for their fabrication.

2 Materials

2.1 Lab-on-a-Chip for High-Throughput Pollen Germination and Guided Pollen Tube Growth

1. 4 inch silicon (Si) wafer.
2. Photoresist SU-8 2005 and SU-8 3025.
3. Photomasks with the profile of the microchannels and the grain reservoirs (e.g. designed in the Siemens NX CAD software and ordered from Selba AG).
4. Spin-coater.
5. Hot plate.
6. Mask aligner.
7. Developer mr-DEV 600.
8. Polydimethylsiloxane (PDMS).
9. Desiccator.
10. Acetone, isopropanol (IPA), deionized (DI) water.
11. Heating oven at 80 °C.
12. Razor blade or box cutter.
13. 1.5 mm biopsy punch.
14. Cover glass (24 mm x 60 mm).
15. Adhesive tape.
16. O_2 plasma asher.

2.2 Lab-on-a-Chip Loading Tool

1. 5 ml syringe (or smaller) with a slip tip.

2. 200 µl plastic pipette tip.

3. 15 cm long silicon tube with an inner diameter of 2 mm.

4. Short plastic tube (6–8 mm long) that fits tightly into the inlet holes of the LOCs (1.5 mm in diameter).

2.3 Pollen Tube Germination and Lab-on-a-Chip Loading

1. Flowering *Arabidopsis thaliana* plants. Wild-type or transformed plants expressing the molecular sensor YC3.60 (*see* **Note 1**).

2. Plant incubator or growth chamber set at 23 °C, 60 % humidity.

3. *Arabidopsis* Pollen Tube Growth Medium (PTGM) based on ***(20)*** (stored at 4 °C, equilibrated to room temperature before experiment): 1.6mM H_3BO_3, 5mM $CaCl_2$, 5mM KCl, 1 mM $MgSO_4$ and 10% sucrose at pH 7.5.

4. Ultra-low-gelling agarose with a melting point ≤60 °C and a gelling point ≤20 °C.

5. 1.5 ml Eppendorf tubes.

6. Thermomixer.

7. Dissecting microscope.

8. LOCs prepared under 2.1.

9. Loading tool made under 2.2.

10. Fridge at 4 °C.

11. Pipette and tips.

2.4 Microscope and Camera Setup

1. Inverted fluorescence microscope with 40x and 60x objective lenses suitable for fluorescence microscopy.

2. Dual CCD camera (alternatively: beam splitter or fast filterwheels) set up for CFP/YFP ratio imaging.

3. Fluorescence LED illumination system equipped with suitable LED Array Modules (LAMs).

4. An *xy* microscope stage actuated by a piezomotor controller with microscopy slide holder.

2.5 Force Measurement and Force Sensor Positioning

1. A force-sensing plate at around the same length scale as the PTs and the microchannels which can be placed less then 1 µm above the cover glass in front of the microchannels of the LOC. The force-sensing direction has to correspond to the direction of PT growth (e.g. parallel to the microchannels). We used a lateral force sensor from FemtoTools AG (www.femtotools.com), offering a 50 x 50 µm force-sensing plate. The standard deviation of the force in our system was 0.18 µN (*see* **Note 2**).

2. 3-axis micropositioner with submicrometer resolution composed of three orthogonal linear actuators and a control unit to either manually control the axes or, connected to a computer, perform automated movements.

3. An adapter arm to mount the force sensor to the micropositioner (*see* **Note 3**).

4. Cable to connect the force sensor to a data acquisition device and to a computer. This connection is used for force readout and powering the sensor.

2.6 Image Processing and Analysis

1. Image processing software such as Fiji with the Bio-Formats plugin (https://imagej.net/Fiji).
2. FRET-IBRA toolkit (https://github.com/gmunglani/fret-ibra).
3. TIGRMUM toolkit (https://github.com/gmunglani/TIGRMUM).

3 Methods

3.1 Lab-on-a-Chip Device Fabrication Using Soft-Lithography (*8*)

3.1.1 Create 2-Layer Mold (1st Layer: Defines Height of Microchannels, 2nd Layer: Defines Height of Grain Reservoirs)

1. Clean Si-wafer (Fig. 2a) with acetone, IPA, and DI water in an ultrasonic bath.
2. Apply an even layer of SU-8 photoresist to the Si-wafer with spin-coating (Fig. 2b). The thickness of the resist defines the height of the microchannels (e.g. 7 µm for *Arabidopsis* with SU-8 2005, *see* **Note 4**).
3. Dry on a hot plate for 2 min 10s at 95 °C (soft baking).
4. The design of the photomask containing the microchannels and the grain reservoirs is transferred into the photoresist using UV exposure (Fig. 2c).
5. Put on a hot plate for 3 min 10 s at 95 °C (post-exposure bake).
6. Develop photoresist in two subsequent baths with developer mr-DEV 600 for 2min 10 s and for 10 s, rinse with IPA and DI water and dry it with N_2 (Fig. 2d).
7. Put on a hot plate for 10 min at 150 °C (hard bake) to reduce stress in resist material.
8. Spin coat a second layer of SU-8 to set the height of the grain reservoirs (e.g. 30 µm for *Arabidopsis* with SU-8 3025, *see* **Note 4**).

9. Repeat Steps 3. to 5. with a photomask containing the reservoirs without the microchannels and different times and temperatures: Soft bake for 11 min 40 s at 95 °C, post-exposure bake for 1 min at 65 °C and for 3 min 40 s at 95 °C, develop for 6 min, and hard bake for 10 min at 150 °C (Fig. 2f).

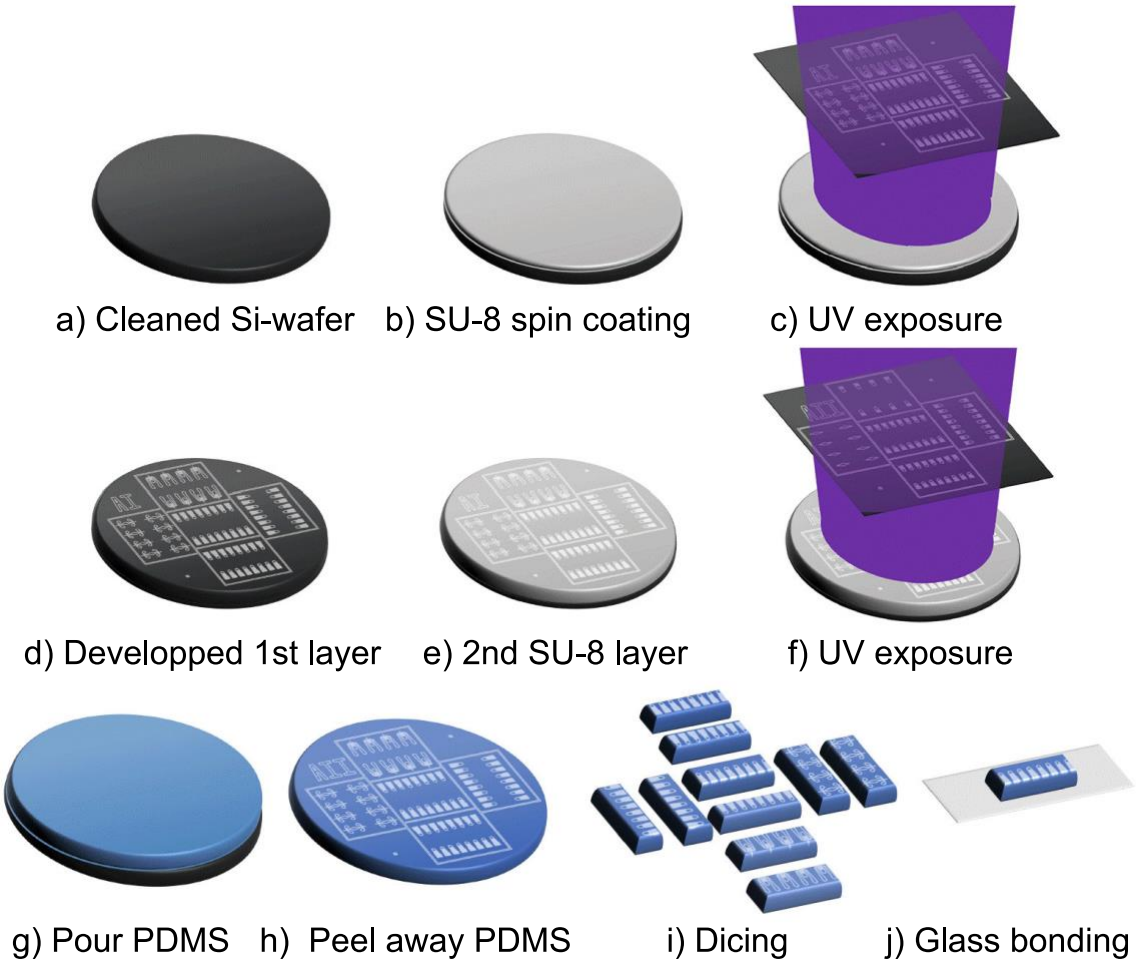

Fig. 2 Lab-on-a-chip fabrication steps. a)-c) First layer of the SU-8 mold, starting with a cleaned chip, which is then covered with SU-8 photoresist. The pattern of the microchannels and the grain reservoir is transferred into the resist with UV-light. d)-f) After developing the first layer, the second layer is deposited by repeating step b) and c) with another SU-8 resist and a mask with the design of the grain reservoirs. g)-h) PDMS is poured onto the mold, cured by heating, and peeled off. i) The PDMS is cut into the desired shapes and j) bonded to separate cover glass slides. (Illustration adapted from (8)).

3.1.2 PDMS Replica Molding

1. Fill 10 parts of PDMS prepolymer with 1 part (in mass) of cure agent in a plastic cup. Stir the PDMS solution well until it looks cloudy and non-transparent.
2. Pour the PDMS mixture onto the mold (Fig. 2g).
3. Degas PDMS in a desiccator.
4. Cure the PDMS for 1 h in a preheated oven at 80 °C.
5. Peel the PDMS from the template and cut it into shape using razor blades or box cutters (Fig. 2h, i, *see* **Note 5**).
6. Punch inlet holes for the grain reservoirs using a biopsy puncher.
7. Clean the PDMS with adhesive tape to remove dust.
8. Expose PDMS chips and cover glasses to oxygen plasma to activate the surface.
9. Place the PDMS chips onto the cover glasses to chemically bond them (Fig. 2j).

3.2 Lab-on-a-Chip Loading Tool Assembly

1. Prepare the pipette tip: cut about 1 cm from the wide end of the 200 µl pipette tip to fit it tightly to the outlet nozzle of the syringe. Cut also about 5 mm from the pointy end to increase the opening diameter.
2. Fit prepared pipette tip to the syringe.
3. Pull silicone tube over the pipette tip as far as you can.
4. Add plastic tube into the other end of the silicone tube such that it protrudes for about 1-2 mm (*see* **Note 6**).

3.3 Pollen Germination and Growth

3.3.1 Prepare PTGM-agarose Aliquots

1. Dissolve 0.8 % (w/v) agarose in PTGM by gentle heating in a microwave. To avoid agglutination, add the agarose after the PTGM.
2. Aliquot into Eppendorf tubes (1 ml per tube).
3. Let the agarose solidify (it can be re-melted at 63 °C for later use).

3.3.2 *Arabidopsis* Pollen Collection and Lab-on-a-Chip Loading

1. Thaw PTGM (without agarose) or prepare freshly as in 2.3.
2. Collect 20-30 open flowers and incubate in moist chamber for ≥30 min.
3. Melt PTGM-agarose aliquot at 63 °C or prepare freshly, see 3.3.1.
4. Equilibrate to 23 °C (*see* **Note 7**).
5. At the same time equilibrate the loading device (syringe, tube, and whatever is used) to 23 °C (*see* **Note 8**).
6. Transfer the flowers to the prepared Eppendorf tube(s) and vortex vigorously. Additionally, shake the Eppendorf tube at maximal frequency in a thermocycler (preheated to 23° C).
7. Spin pollen grains down at 950 x rcf for 4 min.
8. Remove as much of the floating flower debris as possible using tweezers.
9. Carefully load the LOC loading tool with the agarose-pollen mix (avoid air bubbles in the tube).
10. Load each channel system of the LOC (Fig. 3a) by fitting the protruding plastic tube of the loading tool into the inlet hole. Observe the accumulation of pollen grains in the reservoir and, when it is full, carefully remove the loading tool (*see* **Note 9**).

11. Place loaded chip in the fridge at 4 °C for 4 minutes (*see* **Note 10**).

12. Add liquid medium on top of the channels and in front of the channel exits (*see* **Note 11**).

13. Incubate the loaded LOCs in a moisture chamber and place it in a growth chamber at 23 °C under light (*see* **Note 12**) for 3 h (*see* **Note 13**).

14. Transfer LOC to the microscope and check for PTs in the channels to get an overview of the growing PTs and how far away they are from the channel exit (Fig. 3b).

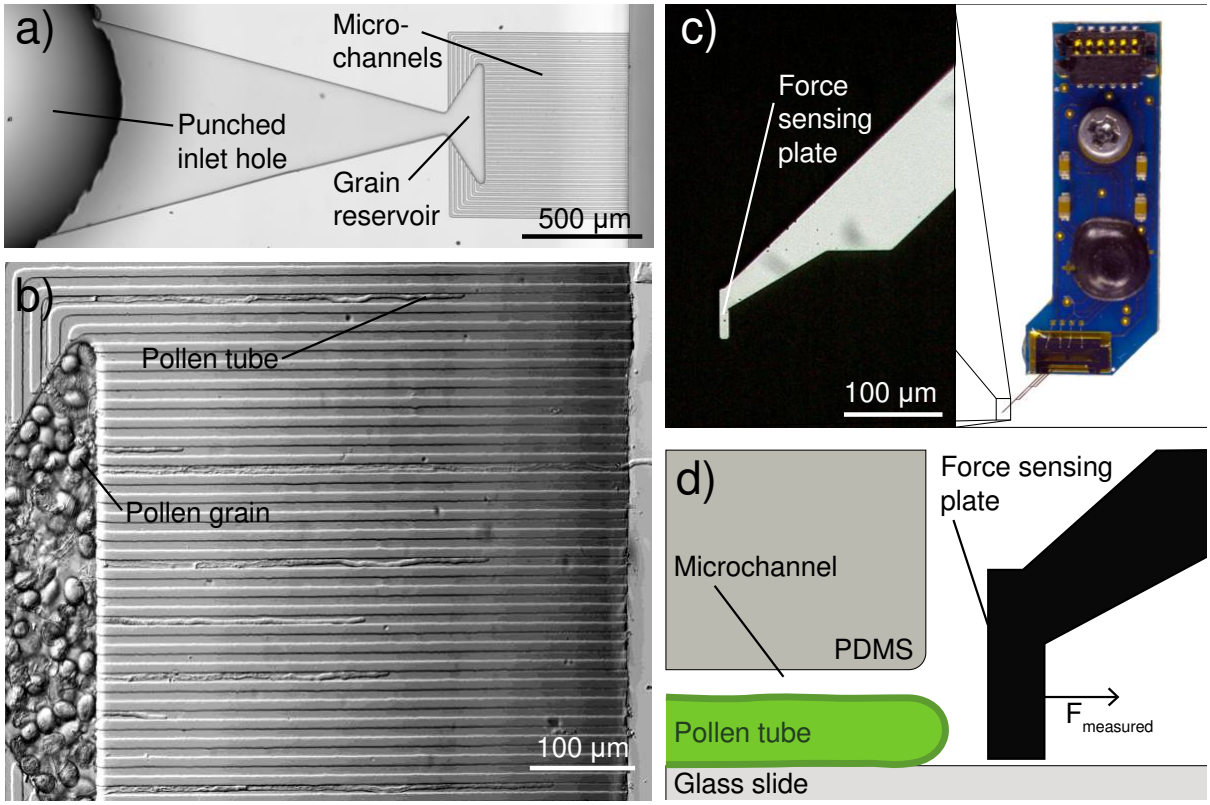

Fig. 3 Experimental configuration. a) The LOC device consists of a grain reservoir and microchannels. Pollen grains are filled into the reservoir through the 1.5 mm inlet. b) Pollen grains germinate inside the reservoir and the protruding PTs grow through the microchannels. c) The MEMS-based capacitive force sensor provides a 50 x 50 µm force-sensing plate. d) The force-sensing plate is placed in front of a microchannel to measure the forces during the interaction of exiting PTs with this microrobotic obstacle.

3.4 Force Measurements and Force Sensor Positioning

1. Mount the 3-axis micropositioner onto the *xy* microscope stage.
2. Power up the microscope stage and the micropositioner.
3. Connect the sensor to the data acquisition device and mount it to the micropositioner.
4. Place the LOC on the microscope holder with the microchannels facing the force-sensing plate of the force sensor.
5. Place the sensing plate roughly 3 mm in front of the PDMS and above the cover glass by manually controlling the micropositioner.
6. Move the force sensor down until the force-sensing plate touches the cover glass (we refer to that as "find contact") and jump back to a safe distance of about 500 µm (*see* **Note 14**).
7. Use the microscope stage to check the LOC for growing PTs close to exit a microchannel (Fig. 3b, *see* **Note 15**).
8. Move the force-sensing plate (Fig. 3c) close to the exit of the microchannel of interest (manually with the micropositioner) (*see* **Note 16**). Find contact with the cover glass and move up 1 µm (*see* **Note 14**). Then fine-adjust the position of the force-sensing plate in front of the exit manually (Fig. 3d, *see* **Note 17**).
9. Start recording the force signal (Fig. 4a) and start fluorescent imaging (see 3.5).
10. Wait till the PT emerges out of the microchannels and interacts with the force-sensing plate (Fig. 4b).
11. Stop recording the force signal after the PT adapted its growth direction (Fig. 4c).
12. Move the sensor plate away from the microchannel and 500 µm up and repeat from step 7 (*see* **Note 18**).

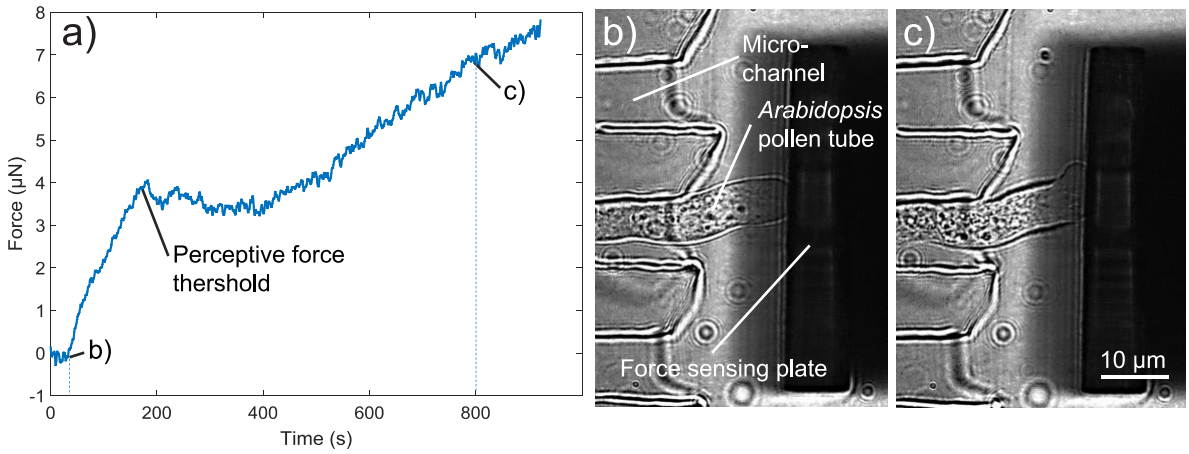

Fig. 4 Typical interaction of a pollen tube with the force-sensing plate. a) Force curve of the PT-obstacle interaction, showing the initial contact and the perceptive force threshold. b) Initial contact of the PT with the plate. c) Change of the PT's growth behavior.

3.5 Microscope and Camera Setup

1. Set up your imaging system for CFP/YFP ratio imaging. Excitation: use LED at 440 nm to illuminate the sample. Emission: use optical block for simultaneous imaging of CFP and YFP fluorescent signals.

2. Set focus carefully with transmitted light illumination and quickly confirm it in the fluorescent channels (*see* **Note 19**).

3. Set the LED power and exposure time to the minimum possible to reduce bleaching and phototoxic damage to the sample. Turn on LEDs only during the exposure time when images are taken or use a shutter (*see* **Note 20**).

4. Take image stacks at 1 Hz or whatever suits your needs.

3.6 Image Analysis

3.6.1 Preparation of Image Stacks for Further Analysis

The FRET-IBRA software needs two TIFF stacks as an input. Depending on your image acquisition software it may require more or less steps to get your raw image data into a useful form. Therefore, some or all of the following steps may be facultative.

1. Start Fiji and import your image stacks via the Bio-Formats Importer plugin.

   If your camera acquires both channels in a single image per time point (i.e. Orca-D2 from Hamamatsu), you have to crop each frame to get individual stacks for the donor and the acceptor channel. Tick the Crop on import option from the import options window and set the coordinates in the following crop options window.
   If your camera acquires individual channels per frame (i.e. Leica systems), tick Split channels from the import options instead.
   If you don't know the stack parameters, you can additionally tick Display metadata in the import options window. Later on, you will need information such as frame rate, resolution and bit depth to configure the FRET-IBRA software. In case, your image acquisition software does not save metadata, you have to take notes of these parameters during the experiment.
2. Trim the stack if you don't want to analyze the entire stack, or if, for example, the focus is lost after some time.

3. Save the donor stack as a TIFF with the name *filename*_donor.tif. Do the same with the acceptor stack (*filename*_acceptor.tif, *see* **Note 21**).

3.6.2 Background Subtraction, Bleaching Correction, and Ratiometric Processing

1. Make yourself familiar with the FRET-IBRA software. A tutorial can be found here (https://github.com/gmunglani/fret-ibra/blob/master/examples/Tutorial.md).

2. Set the *File parameters* in the config file. An example can be found here (https://github.com/gmunglani/fret-ibra/blob/master/ibra/Config_tutorial.cfg). *File parameters* are the *input_path* (the absolute path to where your TIFF stacks are saved), the *filename*, which can be freely chosen, and the range of frames you want to process. For the *frames* parameter you can either choose a continuous range (i.e. 1:10) or individual frames (i.e. 1,5,10). Set the *bit_depth* parameter of your images (8, 12, or 16 bits).

3. Run the *Background subtraction* module by setting the value for the *option* parameter. The values are 0 for the acceptor channel (YFP) and 1 for the donor channel (CFP). The following steps have to be done for both channels separately.

4. Set the *Background parameters.* The size of the moving *window* must be an integer fraction of the image dimension in pixels. For an image of 1280X960 pixels the suggested value is 40. The *eps* value for the DBSCAN clustering algorithm should be set to 0.01 for a start. For more information check the tutorial.

5. Set the frame range (see 3.6.2.2). Especially for very large stacks, it is advisable to choose short ranges at the beginning and at the end of the stack (i.e. 1:20 and end-20:end) to check if the chosen *eps* values are suitable. Batches of discontinuous frames can be processed with different *eps* values, which are then automatically sorted and concatenated together. Overlapping frames are overwritten when re-processed.

6. Run FRET-IBRA with the command ./ibra.py -c <config file path> [options]. Available options can be found in the FRET-IBRA README file or by running ./ibra.py -h.

7. Check the produced video animation for appropriate background subtraction. If it didn't work for several frames, adjust the *eps* value. For an overview of all output files of FRET-IBRA *see* **Note 22.**

8. When suitable *eps* values are found, run the entire range you want to analyze.

3.6.3 Ratiometric Processing

1. Open the config file and activate the *Ratio processing* module by setting the *options* parameter to 2 and changing the values in the *Ratio parameters* section to your needs.

2. Set the *register* and *union* parameters to 1. This will align the two channels and correct for signal overlap, respectively.

3. Check the two graphs that are produced (*filename*_intensity_nonbleach.png and *filename*_pixelcount.png, Fig. 5) for frames that did not work. They are easy to identify by sharp drops in signal intensity (see tutorial).

4. Correct these frames by going back and running the *Background subtraction* module on these frames for both the donor and acceptor stacks.

5. Run the *Ratio processing* module again.

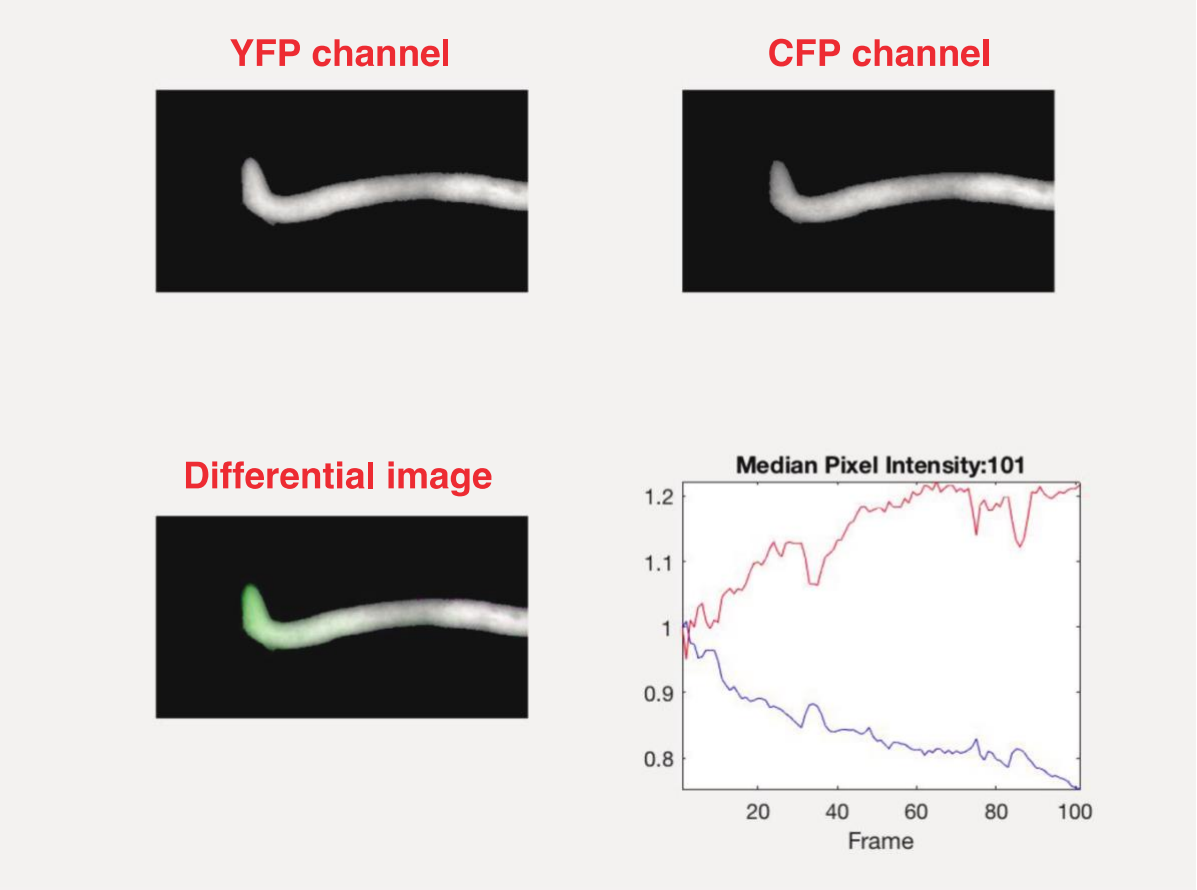

Fig. 5 Output from the FRET-IBRA software. All panels show the same frame (101) of an exemplary pollen tube. The two panels on the top show the YFP and the CFP channels after successful background subtraction. A differential image at the bottom left gives an indication of the quality of the image registration and union of the two channels. The two channels are shown in false colors (YFP: green, CFP: pink). The plot at the bottom right shows the median pixel intensity of the two channels (YFP: red curve, CFP: blue curve). Annotations in red do not appear in the original output.

3.6.4 Bleaching Correction (Optional)

1. Open the config file once more and activate the *Bleach correction* module if one or both channels showed strong bleaching (*see* **Note 23**) by setting the *options* parameter to 3.

2. Choose the frame range to which to fit the bleaching effect (*see* **Note 24**). This can be done individually for the donor and acceptor stacks.

3. Set the *fit* parameter to either linear or exponential. This will define the type of regression that is fit to the chosen signal range.

3.6.5 Tip Tracking, Ratio Quantification and Pollen Tube Feature Analysis

1. The TIGRMUM package can be downloaded at *github.com/gmunglani/TIGRMUM*. It does not require any installation and can be run directly from within MATLAB.

2. The *path* and filename (*fname*) of the input files must first be set. This is followed by the start (*stp*) and end (*smp*) frame numbers of the input TIFF stack on which the algorithm should be run (*see* **Note 25**).

3. The output analysis options must then be set. This includes *tip_plot,* which outputs an animation of the frame by frame tip position named *filename*_growth.avi (Fig. 6a), *video_intensity,* which outputs a normalized pixel-intensity distribution animation named *filename*_ratio.avi (Fig. 6d), *distributions*, which displays histograms of the pixel intensities of the ROI and full pollen tube, and *workspace*, which saves the output in a HDF5 file named *filename*_result.h5.

4. The *tip_detection* parameter then needs to be set (default: 0.5). This is a ratio parameter to aid in tip detection by weighing the ellipse method and morphological thinning (0 is only thinning and 1 is only ellipse).

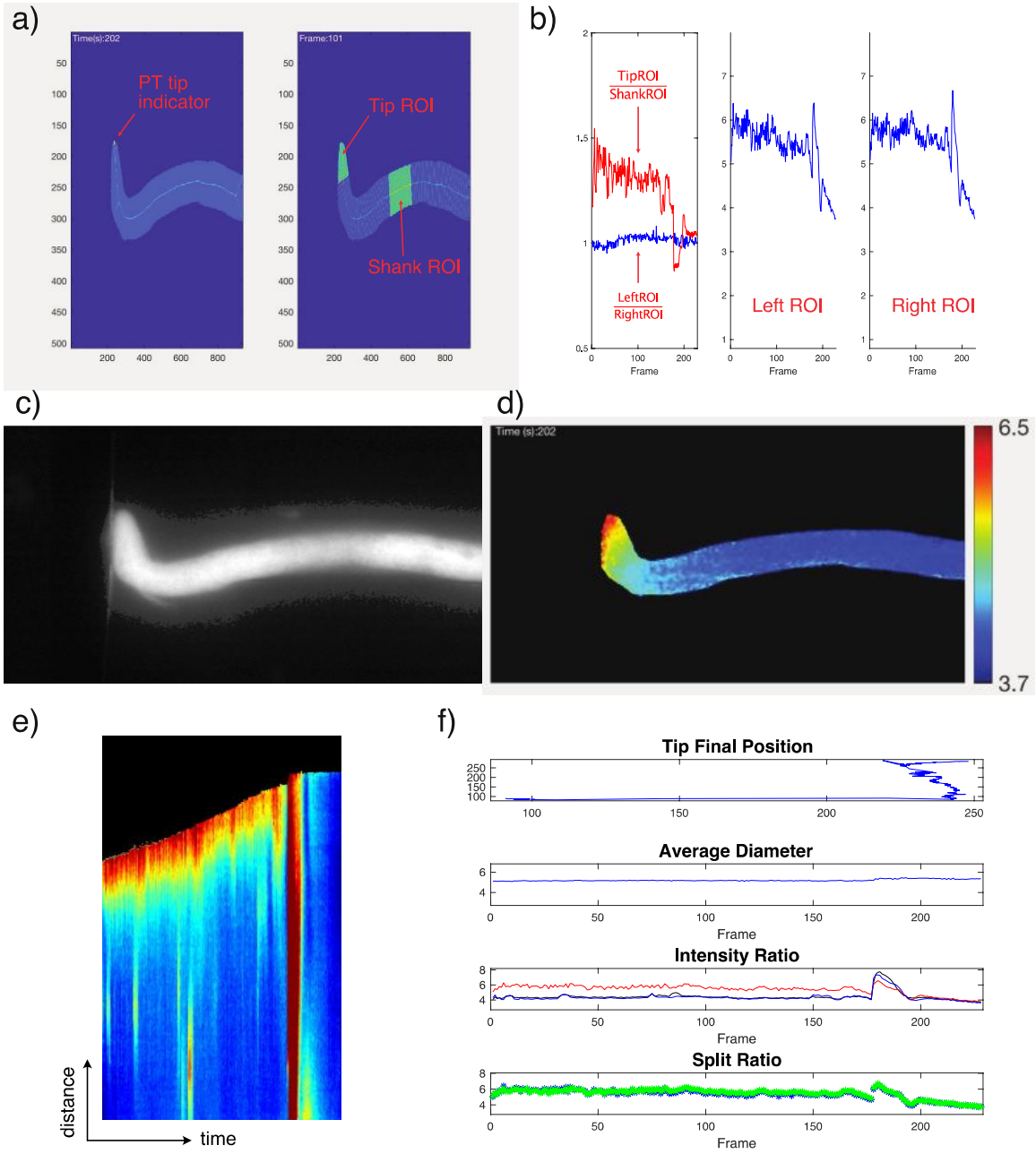

Fig. 6 Output of the TIGRMUM toolkit. a) A control video to check the correct localization of the PT tip (left panel) and the correct position of the chosen Regions Of Interest (ROIs, right panel). b) Quantification of the signal intensities within the ROIs. In this example the Tip ROI was split along the central axis of the PT, such that the left and right halves can be quantified separately (middle and right panel, respectively). The left panel shows the ratio between the Tip ROI and the Shank ROI (red curve), indicating that there is a tip-focused Ca^{2+} gradient. The blue curve represents the ratio between the Left ROI and the Right ROI at the tip. A slight shift in signal intensity towards the left is seen. c) The PT growing along the sensor plate after a change in growth direction (YFP channel). d) The ratiometric image of the situation in c) showing a shift in the tip focused Ca^{2+} gradient towards the sensor plate. e) The kymograph of the ratio image along the center line of the pollen tube, again showing the tip-focused gradient and a huge Ca^{2+} burst towards the end before PT growth stops. d) The toolkit delivers additional data such as tip position, the average diameter along the entire PT over time, the signal intensity of the YFP and CFP channels, and the average ratio of the whole PT (Intensity Ratio panel) as well as the ratio of the Left ROI and the Right ROI (Split Ratio). Annotations in red do not appear in the original software output.

5. The region of interest options then need to be stated. These include *ROI_type* (0, 1, or 2 for No ROI, Moving ROI, and Stationary ROI), *split*, which splits the ROI along its center line to measure the ratio of the intensities of right and left halves of the pollen tube, *circle*, which indicates a circle ROI as a fraction of the pollen tube diameter, *starti* and *stopi*, which prescribes the geodesic distance along the center line of the pollen tube that the ROI is created for, and *pixelsize*, which gives the pixel to μm conversion of the image.

6. Finally, the *Cmin* and *Cmax* parameters indicating the normalization scale of the pixel values for visualization need to be set, along with the averaging width (*nkymo*) of the output kymograph, *filename*_kymo.png (Fig. 6e).

7. A diameter measurement animation named *filename*_diameter.avi, the ratio of the pixel intensities between both halves of the ROI named *filename*_ROI.avi, and the average pixel intensity per frame named *filename*_pixel.avi are also included in the output.

4 Notes

1. The method described here works essentially with any kind of cytoplasmic fluorescent reporter.

2. The advantage of the lateral force sensor provided by FemtoTools AG is that the force sensing plate is far away from the rest of the sensor. A silicon arm (around 4 mm long and 500 μm thin) connects the force sensing plate to the sensor at an angle of 45°. In this way, the placing of the sensing plate is not hindered by the base of the sensor (including integrated circuit board) and can be as close to the exit of the microchannels as wanted. Furthermore, the risk of crashing the sensor into the cover glass is minimized.

3. The sensor is connected to the micropositioner using an adapter arm. The design of the adapter arm can be changed according to the application. If the arm is too long

(> 20 cm), the position of the sensing plate slightly changes over time, which can influence the force measurement. The arm should be as short as possible to get a stable position of the sensing plate during a single PT experiment (up to 30 min). The arm can be fabricated with a laser cutter (acrylic), with a CNC machine (metallic), or with a 3D printer.

4. The thickness varies with the type of SU-8 used and the revolution per minute (rpm) of the spin-coater. There are look-up sheets to find the optimal combination for the desired thickness (e.g. SU-8 2015: http://microchem.com/pdf/SU-82000DataSheet2000_5thru2015Ver4.pdf, SU-8 3025, http://microchem.com/pdf/SU-8%203000%20Data%20Sheet.pdf).

5. The cuts in front of the microchannels have to be very straight or even a little bit tilted backwards, such that the angle between the glass slide and PDMS cut is equal or smaller than 90 degrees. If the angle between glass slide and PDMS cut is obtuse, the top part of the PDMS will be overhanging, which makes it impossible to bring the force-sensing plate close to the exit of the microchannels. It further increases the risk of damaging the sensor, e.g. by crashing into the PDMS when moving up.

6. The plastic tube should be long enough to fit into the inlet hole but only as long as to allow the silicon tube to sit on the PDMS and seal the system tightly.

7. The equilibration (cooling) of the re-melted agarose in a thermocycler takes quite some time. Start at least 1 hour before LOC loading.

8. If two incubation time points are planned, equilibrate an empty Eppendorf tube to 25 °C and split the agarose between the two Eppendorf tubes.

9. Excess PTGM exits from the channel openings. These drops can be left to solidify and later on being carefully removed with a toothpick without disturbing the channel openings. You need to take care not to leave traces of agarose in front of the channels since this can interfere with sensor positioning.

10. Be careful. Keeping the LOCs in the fridge for too long can delay the germination for hours!

11. Medium on top of the channels needs to be largely but not completely removed for bright-field microscopy. Otherwise, the light scattering will greatly affect the quality of the images.

12. We used an incubator for *Arabidopsis* PT growth during "day time". Could also work in the dark but we have never tried it.

13. Depends on conditions and needs to be tested empirically.

14. A simple LabView™ program is used to move the sensor down at a constant velocity while the signal from the force sensor is read out. The sensor has a small cross sensitivity and slightly reacts to forces applied parallel to the force plate. When the bottom of the force-sensing plate touches the glass slide and the signal increases above a set threshold, the motion is stopped and the micropositioner moves the sensor to a predefined position above the cover glass (e.g. 500 µm, 1 µm).

15. The micropositioner moves along with the microscope stage since it is mounted on it. Therefore, the relative position between LOC and force sensor does not change while checking the entire LOC for suitable PTs using the microscope stage, avoiding the sensor tip to crash into the PDMS structure.

16. For safety reasons, we move the sensor plate roughly 500 µm above the glass slide before moving it in *x* and *y* direction in order not to crash into obstacles that can lay on top of the cover glass (e.g. pollen grains). However, the sensor can still hit the PDMS structure (height ~3 mm).

17. Sometimes there is some agarose in front of the channel exit, which would influence the force measurements. By moving the plate back and forth along the PDMS chip in front of the microchannels, the agarose can be "plowed" away from the exit of the microchannels.

18. Repeat as long as there are promising PTs in the channels. Make sure that the media reservoirs on top and in front of the channels do not dry out by adding fresh medium when necessary. Once in a while, e.g. if no suitable PTs are ready for measuring, put the LOC back into the moist chamber and replace the liquid reservoirs on top and in front of the channels.

19. Should the sample get out of focus while filming, do not try to readjust the focus because the FRET-IBRA software will have problems to deal with this later on. Instead, stop the video, readjust the focus, and start a new stack.

20. Use the CFP channel as a reference. If you can just see the signal, the FRET-IBRA software can extract it. If the software has problems discriminating between signal and background, increase exposure by either increasing LED power or exposure time.

21. Replace "*filename"* with any name of your choice, but it must be the same for the donor and acceptor stack, respectively. The "_acceptor.tif" and "_donor.tif" parts of the stack name cannot be changed.

22. Output variable names in FRET-IBRA.

    # Animation of background subtraction.
    *filename*_acceptor_frames22_28.avi (frames22_28 indicate the frame range).
    *filename*_donor_frames22_28.avi

    # TIFF stacks and h5 after background subtraction.
    *filename*_acceptor_back.tif
    *filename*_donor_back.tif
    *filename*_back.h5

    # TIFF stack and h5 after ratio processing.
    *filename*_back_ratio.h5
    *filename*_back_ratio.tif

    # Median intensity after ratio processing.
    *filename*_intensity_nonbleach.png

    # Pixel count after ratio processing.
    *filename*_pixelcount.png

    # TIFF stack after bleaching (h5 is same as before).

*filename*_back_ratio_bleach.tif

Median intensity after bleaching.
*filename*_intensity_bleach.png

Log file.
filename.log

23. If both channels bleach at the same rate, the ratio is not affected. In our hands, however, the YFP channel bleaches at a different rate than the CFP channel, which leads to an apparent decrease in the ratio. This can be corrected for by running the *bleach* module.

24. Bleaching for the YFP channel is usually pretty strong at the beginning of the exposure. Sometimes it is a good option to fit the bleach effect only to this region instead of the entire stack.

25. The input stack can be any TIFF stack produced by FRET-IBRA. If the quality is good enough, TIGRMUM works also on non-background-subtracted TIFF stacks.

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